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4-Methoxy-3-hydroxyphenylglycol as an Internal Standard for the Determination of 3-Methoxy-4-hydroxyphenylglycol in Urine: Results Obtained in Depressed Patients and Healthy Controls

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Summary: A modification of the method developed by *Dekirmenjian & Maas* ((1970) Anal. Biochem. *35*, 113–122) is described for the determination of 3-methoxy-4-hydroxyphenylglycol in urine. The use of 4-methoxy-3-hydroxyphenylglycol as an internal standard improves the accuracy, simplicity and reproducibility. Therefore, the method is suitable for routine determination in laboratories without gas chromatography/mass spectrometry equipment. Some results obtained in depressed patients and healthy controls are presented.

4-Methoxy-3-hydroxyphenylglykol als interner Standard für die Bestimmung von 3-Methoxy-4-hydroxyphenylglykol im Harn:
Ergebnisse bei Patienten mit Depression und gesunden Kontrollpersonen

Zusammenfassung: Zur Bestimmung von 3-Methoxy-4-hydroxyphenylglykol im Harn wird eine Modifikation der Methode von *Dekirmenjian & Maas* ((1970) Anal. Biochem. *35*, 113–122) beschrieben. Die Verwendung von 4-Methoxy-3-hydroxyphenylglykol als internem Standard verbessert Richtigkeit, Handhabung und Reproduzierbarkeit. Daher ist die Methode für Routinebestimmungen in Laboratorien ohne Gaschromatographie/Massenspektrometrie-Ausrüstung geeignet. Einige Ergebnisse von Patienten mit Depression und gesunden Kontrollen werden mitgeteilt.

Introduction

The major metabolites of norepinephrine in urine are 3-methoxy-4-hydroxymandelic acid and 3-methoxy-4-hydroxyphenylglycol. It is assumed that the aldehydes produced in the metabolism of norepinephrine follow a predominantly reductive pathway to the glycol in the central nervous system (2). The average contribution by brain to the total body production of 3-methoxy-4-hydroxyphenylglycol is estimated to be 63% in man (3).

The value of 3-methoxy-4-hydroxyphenylglycol determinations for the elucidation of the aetiology of depressive illness is doubtful, but they may be useful for classifying depressive disorders and predicting drug response (4, 5).

Materials and Methods

Chemicals and solutions

1.0 mol/l Acetate buffer pH 6.0
20 g/l EDTA
$Na_2S_2O_5$ p. A.
1.0 mol/l $KHCO_3$
NaCl p. A.
Chloroform p. A.
Ethyl acetate p. A. freshly distilled over K_2CO_3
Pyridine p. A.
Toluene p. A. (all Merck, Darmstadt)
β-Glucuronidase/Arylsulfatase, EC 3.2.1.31/3.1.6.1 (Boehringer, Mannheim)
Pentafluoropropionic anhydride (Pierce, Rotterdam)
3-Methoxy-4-hydroxyphenylglycol piperazine salt (EGA-Chemie, Steinheim)
4-Methoxy-3-hydroxyphenylglycol (R. Paesel KG, Frankfurt)
Dimethyldichlorosilane (Fluka, Neu-Ulm)

0340-076X/80/0018-0579$02.00

Equipment

Gas chromatograph: Hewlett-Packard 57 30A (ECD, constant current and variable pulser frequency)

Automatic sampler: Hewlett-Packard 76 71A

Integrator: Hewlett-Packard 33 80A

Treatment of glassware

4 ml glass-stoppered tubes for derivatisation should be silanized with a solution of 50 g/l dimethyldichlorosilane in toluene and then dried overnight at 300 °C. Silanisation should be repeated every month.

Procedure

Urine sampling

Two or three 24-h urine samples were collected from untreated in-patients or healthy volunteers. Patients had to meet the criteria for primary affective disorders as described by *Feighner* et al. (6). The criterion for bipolarity was a prior history of hypomania or mania. After determination of pH and creatinine (according to the method of *Jaffé*) 0.5 g/l sodium disulfite was added and aliquots were stored at – 20 °C.

Incubation

Since more than 90% of urinary 3-methoxy-4-hydroxyphenylglycol is present in the form of the glucuronide and the sulphate (10), extraction had to be preceded by enzymic hydrolysis.

0.2 ml 1.0 mol/l acetate buffer, 0.1 ml 20 g/l EDTA and 0.2 ml β-glucuronidase arylsulfatase were added to 0.5 ml urine and incubated at 37 °C for 24 h. 0.1 ml of a solution of 0.02 g/l 4-methoxy-3-hydroxyphenylglycol in acetate buffer pH 6.0 were added to the hydrolyzed mixture.

In experiments according to procedure a) (described below), increasing amounts of a solution of 0.01 g/l 3-methoxy-4-hydroxyphenylglycol (free acid) in acetate buffer pH 6.0 were also added.

Extraction

The mixture was extracted twice with 2 ml $CHCl_3$ and the organic layer discarded. After addition of 200 mg NaCl, the aqueous phase was extracted with 2 ml ethyl acetate, while shaking during one minute on a Vortex mixer. After centrifugation, the organic phase was carefully removed with a *Pasteur* pipette. The extraction was repeated three times. The combined organic layers were brought to 2 ml at reduced pressure with a rotary evaporator and then washed with 1 ml 1 mol/l $KHCO_3$. The $KHCO_3$ layer was re-extracted with 0.5 ml ethyl acetate then discarded. The organic phases were transferred into the silanized vials and dried under a stream of nitrogen. (Extracts may be stored at – 20 °C for some days, provided that they are protected from moisture.)

Derivatisation

To the vial containing the extract, 0.04 ml of a mixture of pyridine/ethylacetate 1:2000 and 0.05 ml pentafluoropropionic anhydride was added, the vial was stoppered and the mixture allowed to react at + 25 °C for one hour. Then, the contents of the vial were dried under a stream of nitrogen. Since the derivate is volatile, the nitrogen-stream must be carefully regulated and stopped immediately after drying. The residue was dissolved in 1 ml ethyl acetate.

Chromatography

1 µl of the sample was injected twice on a coiled glass column 10 ft × 4 mm internal diameter, packed with 3% OV-210 on gaschrom Q 80–100 mesh. The injection port temperature was 250 °C, the temperature of oven and detector 170° and 300 °C, respectively. Gas flow rate (argon-methan 95:5) was 40 ml/min.

Evaluation

Two methods were used:

a) 5 samples of each urine specimen were hydrolyzed, and in addition to a constant amount of 4-methoxy-3-hydroxyphenylglycol (2000 ng), increasing amounts of 3-methoxy-4-hydroxyphenylglycol were added to 4 of them (200, 400, 600, 1000 ng). From the ratios of peak areas (3-methoxy-4-hydroxyphenylglycol/4-methoxy-3-hydroxyphenylglycol) a regression line was constructed. The intersection with the abscissa gave the amount of 3-methoxy-4-hydroxyphenylglycol in the sample without additional 3-methoxy-4-hydroxyphenylglycol (fig. 1). r is a measure of the accuracy of the determination. Our values varied from 0.97 to 1.00.

b) From each urine specimen, only 2 samples were hydrolyzed and only 2000 ng 4-methoxy-3-hydroxyphenylglycol added. The resulting peak area ratios correspond to the ratios 3-methoxy-4-hydroxyphenylglycol/4-methoxy-3-hydroxyphenylglycol. A calibration curve (established by adding varying amounts of 3-methoxy-4-hydroxyphenylglycol and a constant amount of 4-methoxy-3-hydroxyphenylglycol to *Ringer* solution and treating it in the same way as an urinary sample) showed that for example 2000 ng 3-methoxy-4-hydroxyphenylglycol (corresponding to 2 ng injected) and 2000 ng 4-methoxy-3-hydroxyphenylglycol (corresponding to 2 ng injected) resulted in a ratio of 1.0 (fig. 2).

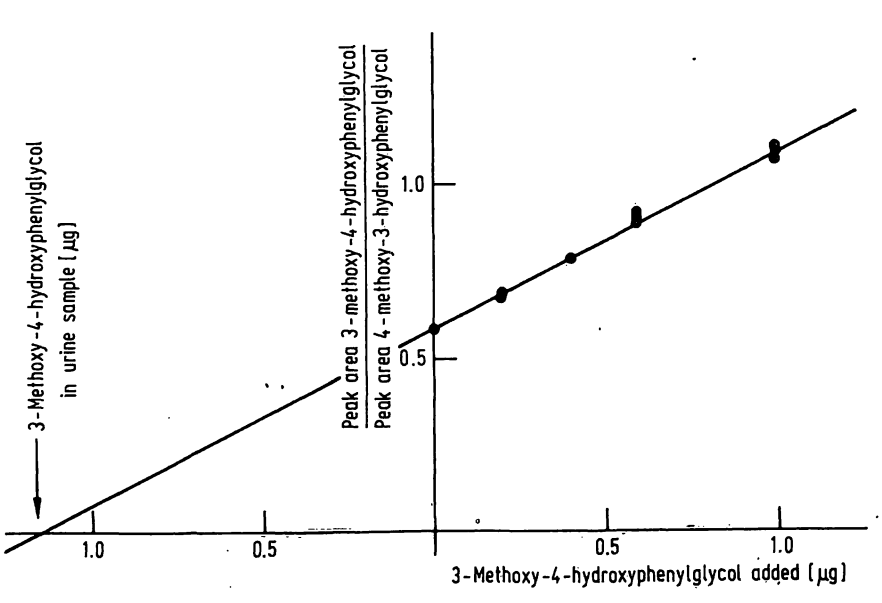

Fig. 1. Regression line for the determination of 3-methoxy-4-hydroxyphenylglycol in a urine sample according to method a).

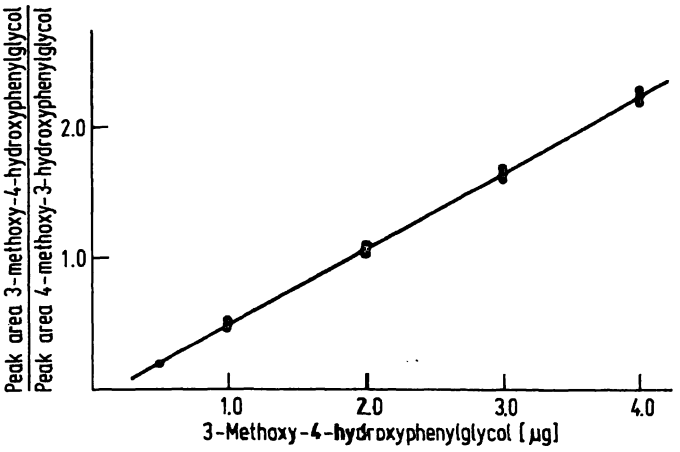

Fig. 2. Calibration curve for the determination of 3-methoxy-4-hydroxyphenylglycol, using 4-methoxy-3-hydroxyphenylglycol as internal standard.

With lower amounts of 3-methoxy-4-hydroxyphenylglycol the ratio, counts 3-methoxy-4-hydroxyphenylglycol/counts 4-methoxy-3-hydroxyphenylglycol, tended to be lower than expected; with higher amounts of 3-methoxy-4-hydroxyphenylglycol, it tended to be higher than expected. The deviation was not great, but constant over a series of calibration curves. The reason for this is the loss of a constant amount that is more noticeable in smaller samples. This loss occurred on the column, as could be demonstrated by injection of pure substances. To solve this problem, we always used calibration curves for the calculation.

Results and Discussion

The conditions for the enzymic hydrolysis are well described by *Dekirmenjian & Maas* (1) and only a few parameters were checked by us.

Repeated analysis of the same urine over a period of five months proved that storage is possible without deterioration ($\bar{x}$ = 1439 µg/24 h, s_x = 66, n = 9). The values showed no tendency to decrease.

The extraction with $CHCl_3$ reduced the number of additional peaks. The loss of substance due to $CHCl_3$ extraction was less than 1%. The addition of NaCl led to a better separation of phases and more efficient extraction. Exact timing during the extraction (vortexing and centrifugation) was necessary for reproducible results. The extraction with $KHCO_3$ resulted in lower recovery, but chromatograms showed fewer interfering peaks and a straight baseline.

The derivatisation reaction according to *Fellows* et al. (7), *Halaris* (8) and *Sharpless* (9), using temperatures between 50–65 °C led to erratic results in our hands. More reproducible results were obtained when the reaction was carried out at room temperature, according to *Dekirmenjian & Maas* (1). The addition of pyridine further reduced the variability. The loss of the volatile derivate during drying under nitrogen was very difficult to control.

In our opinion, all these problems can only be solved satisfactorily by using an internal standard the chemical structure of which guarantees a behaviour during the analysis identical to that of the compound to be determined. External standardisation or use of other chemically unrelated compounds as internal standards makes the procedure too susceptible to little variations and precludes routine use.

Besides the OV-210 column, we also used the OV-17 column (*Dekirmenjian & Maas* (1)). On this column, a separation of the two isomers was also possible, but in urine extracts the peaks occurred earlier and were located in a region of impurities. Figure 3 shows a chromatogram on the OV-210 column.

For method a), we found a coefficient of variation of 11.3%. Five determinations of the same urine, each containing increased amounts of 3-methoxy-4-hydroxyphenylglycol, resulted in $\bar{x}$ = 2295 µg/24 h, s_x = 259. Five single determinations of the same urine according to method b) (only 4-methoxy-3-hydroxyphenylglycol added) resulted in $\bar{x}$ = 2323 µg/24 h, s_x = 155. 10 determinations in duplicate with another urine, according to method b) resulted in $\bar{x}$ = 1845 µg/24 h, s_x = 32, coefficient of variation 1.7%. Both methods gave almost equal results. However, in method b) the coefficient of variation was somewhat lower. We therefore used method b) in our subsequent determinations; method a) is not practicable. All determinations were carried out in duplicate with a good correlation between the two assays (r = 0.99, n = 197).

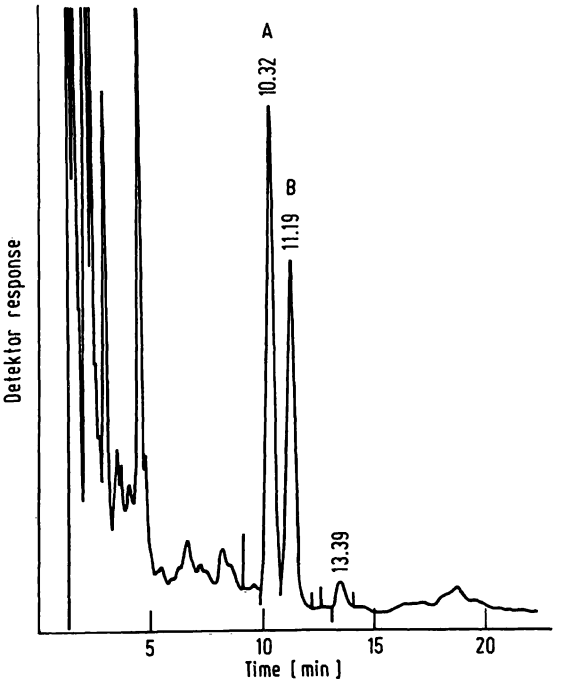

Fig. 3. Typical gas chromatogram of an extract of 0.5 ml urine. According to the calibration curve (Fig. 2), the sample contained 1.83 mg/l 3-methoxy-4-hydroxyphenylglycol.
A = 3-methoxy-4-hydroxyphenylglycol
B = 4-methoxy-3-hydroxyphenylglycol.

Tab. 1. Mean 3-methoxy-4-hydroxyphenylglycol excretion in patients and controls reported in the literature. ♂ = male, ♀ = female, s = single episode, r = recurrent, e = endogenous, ne = nonendogenous, values in μg/24 h.
Own results: Values are also expressed in μg/mg creatine (values in brackets). n = number of individuals. For each individual 2 to 3 separate 24 h urine samples were analysed in duplicate.

	Controls	Depressed patients (mixed group)	Patients with primary affective disorder	Unipolar depressives	Bipolar depressives	Schizoaffective patients
Karoum et al., 1973 (10)	1863 (♂) n = 9					
Shaw et al., 1973 (11)				1401 (♀) n = 22		
Maas et al., 1974 (12)	1674 (♂) n = 19 1348 (♀) n = 21	1394 (♂) n = 20 1155 (♀) n = 48				
Beckmann et al., 1975 (13)				1860 (♀) n = 2	1590^{II} (♀) n = 3 830^{I} (♀) n = 5	
Deleon-Jones et al., 1975 (14)	1348 (♀) n = 21	1137 (♀) n = 33	1032 (♀) n = 21	1161^{s} (♀) n = 14 1207^{r} (♀) n = 13	916 (♀) n = 5	
Josef et al., 1976 (15)	2740 n = 13					
Sharpless, 1977 (9)	2105 (♂) n = 6 1618 (♀) n = 5	2019 (♂) n = 10 1357 (♀) n = 10				
Hollister et al., 1978 (16)	2254 (♂) n = 11 1591 (♀) n = 6					
Pickar et al., 1978 (17)	1630 (♀) n = 5		1184 (♀) n = 10			
Schildkraut, 1978 (4)				1950^{e} n = 16 1814^{ne} n = 13	1209 n = 12	1149 n = 4
Taube et al., 1978 (18)	1029 (♀) n = 10		791 (♀) n = 14			
Goodwin & Potter, 1979 (19)	1830 n = 18	1658 n = 27	1682 n = 23	1930 n = 13	1360 n = 10	1520 n = 4
Modai et al., 1979 (20)			1310 (♀) n = 13		1310 (♀) n = 1	
Own results	2001 (♂) n = 11 s_x = 462		2009 (♂) n = 10 s_x = 556	2166 (♂) n = 7 s_x = 542	1339 n = 7 s_x = 499	
(μg/mg creatinine)	(1.19; s_x = 0.25)		(1.36; s_x = 0.50)	(1.56; s_x = 0.44)	(1.17; s_x = 0.39)	
	1646 (♀) n = 9 s_x = 379		1512 (♀) n = 39 s_x = 526	1558 (♀) n = 35 s_x = 521		
(μg/mg creatinine)	(1.57; s_x = 0.46)		(1.41; s_x = 0.45)	(1.42; s_x = 0.46)		

The time required for analyzing eight urine specimens was about 48 h (using the automatic sampler), including incubation.

For validation of our results, we compared them with data found in the literature (see tab. 1). We also found the sex difference in total 3-methoxy-4-hydroxyphenylglycol excretion per 24 h in patients and controls. In patients, the difference was significant ($p < 0.01$), whereas in controls we only observed a trend ($p < 0.1$). This difference disappears when values are expressed as μg 3-methoxy-4-hydroxyphenylglycol/mg creatinine. 3-methoxy-4-hydroxyphenylglycol excretion in bipolar patients (males and females) was lower ($p < 0.05$) than in unipolar patients, as reported by other authors (4, 13, 14, 19). This also applies only for the total 3-methoxy-4-hydroxyphenylglycol excretion, but not for 3-methoxy-4-hydroxyphenylglycol/creatinine.

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